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14. ABSTRACT

Telomerase activity is associated with over 90% of human breast cancers and is necessary for continued tumor cell growth, making it an ideal target for inhibition therapy. However, pharmacologic inhibitors of telomerase have not been as effective as expected. As such, our objective here is to identify novel telomerase interacting proteins and define their functional relationship to telomerase in order to provide additional targets for telomerase inhibition in breast cancer. In addition to the results that we reported in previous annual reports concerning telomere binding proteins and chaperone interactions, we have found that telomerase is a modified protein, capable of being ubiquinated and sumolylated and is able to be degraded via both nuclear and cytoplasmic mechanisms. We show that inhibition of the Hsp90 chaperone results in telomerase degradation in the nucleus, but association of wild-type telomerase with a dominant-negative version results in cytoplasmic degradation. We show that telomerase is associated with the proteosome in both the nucleus and cytoplasm and that this alternative regulation of telomerase is key for functionally blocking its activity as an adjuvant target for chemotherapeutics for breast cancer.

15. SUBJECT TERMS
telomerase, chaperones, proteomics, immunoprecipitation, regulation

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Table of Contents

Introduction

Telomerase is a cellular reverse transcriptase that is associated with over 90% of human breast cancers and is composed of 2 integral components, an RNA template (hTR - human Telomerase RNA) and a catalytic polymerase (hTERT - human TElomerase Reverse Transcriptase) (**Weinrich et al 1997**). Telomerase is an obvious chemotherapeutic target (Shay and Bacchetti, 1997). Telomerase activity requires its two core components, hTERT and hTR, to be assembled into a functionally active enzyme by the Hsp90 chaperone complex (Holt et. al., 1999). We have previously demonstrated that chaperones are essential for optimal telomerase assembly *in vitro* (**Holt et. al., 1999**) and that Hsp90 itself remains associated with the functional telomerase complex (**Forsythe et. al., 2001**) (see Figure 1).

In a human cancer progression model, increased assembly of telomerase by chaperones, including Hsp90, has been shown to correlate with cancer progression, which is defined as increased aggressiveness *in vivo* (**Akalin et. al., 2001**). These findings indicate that increased expression of the Hsp90 chaperone complex with the associated activation of telomerase activity may be important steps in cancer formation (**Holt et. al., 1999; Akalin et. al., 2001**). While telomerase in cancer progression has been widely studied (reviewed by Shay and Bacchetti, 1997), the role of chaperones in carcinogenesis and their interplay between telomerase and its substrate, the telomere, are less well defined.

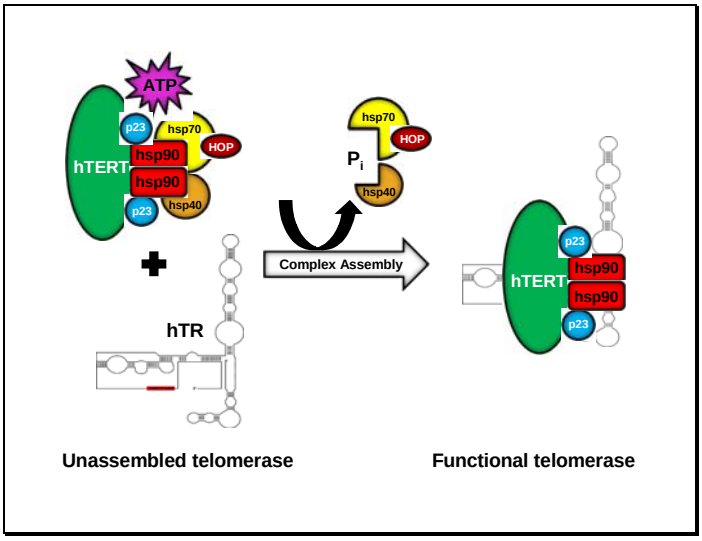

Figure 1. The hsp90 complex is required for assembly of active telomerase. Our working model for the chaperone-mediated ordered assembly of active human telomerase. [hTR - human telomerase RNA; hTERT - human telomerase reverse transcriptase]

Body

Rationale: The overall goal of this proposal has been to identify mammary-specific telomerase interacting proteins that will serve as targets for direct and indirect inhibition of telomerase and determine the consequences of these interactions during breast cancer progression. Given some of the limitations of telomerase reagents, we have slightly altered our goals to include the regulation of telomerase through its interaction with the hsp90 chaperone complex and the association of telomerase with the proteosome machinery. The experiments discussed in this final report will allow further understanding and characterization of the mechanisms of telomerase/telomere structure

and function as it relates specifically to breast cancer and will facilitate innovative techniques and protocols for early detection and treatment of breast cancer.

***Objective #1*: Define the regulation of telomerase by the identification of mammary-specific telomerase interacting proteins using a proteomic approach.**

The previous report indicated problems with hTERT antibodies (still a problem) and that we have been assessing proteins that were bound to hsp90 in breast tumor cells as an alternative to finding telomerase interactors specific for mammary-derived cells. We have found that the hsp90 chaperone complex binds specifically to telomeres and to the telomere binding protein, TRF-2. We continue with this project in an attempt to show a functional significance for this interaction and to determine if chaperones interact with other telomere binding proteins.

During the final funding phase of the proposal, we have found that telomerase is associated with the proteosome machinery, suggesting that telomerase is readily degraded, depending on the state of the cell. As such, we have discovered that telomerase is degraded in the nucleus and in the cytoplasm, using proteolytic mechanisms in both areas of the cell. Nuclear degradation is understudied and represents an important, novel mechanism of telomerase regulation.

Nuclear Degradation

Initial characterization of the MCF-7/GFP-hTERT cell line was performed in terms of Western blot, confocal fluorescence, growth assay and telomere length assays to assess molecular weight, localization, and in vivo activity. GFP-hTERT was shown to be expressed at it's predicted molecular weight of 160 kD with increased telomerase activity and telomere lengths (not shown). The elevated activity and telomere lengths did not affect the growth rate such that the population doubling rate of 0.9 pd per day is similar to control. The nuclear localization and mostly nucleolar exclusion of GFP-hTERT is similar to what has been observed in the literature (Wong et al., 2002) (Fig. 2 left panel).

When treated with a variety of drugs, the GFP-hTERT cells showed a pronounced effect after Radicicol (RAD) treatment, which is an hsp90 inhibitor (Compton et al., 2006). Subsequent drug experiments were done using RAD because chronic blockage of hsp90 in cancer cells causes a transient decline in telomerase activity coupled with reactive oxygen-induced telomere shortening (Compton et al., 2006). To further understand the effect of Hsp90 inhibition, acute and chronic treatments were analyzed for fluorescence and telomere length. Acute exposure caused an almost complete loss of fluorescence within 2 hours (Fig.2 middle picture and quantitation). After 2 days, the fluorescence GFP signal had not recovered but telomere lengths remained the same (data not shown). Even so, the cells continue to divide but at a reduced growth rate of 0.6 PD per day during treatment. With continued hsp90 inhibition on a 2-day cycle, the 8^{th} day analysis showed a recovery of fluorescence, and after 12 and 24 days, the

fluorescent signal was almost fully recovered despite continued presence of Radicicol (not shown).

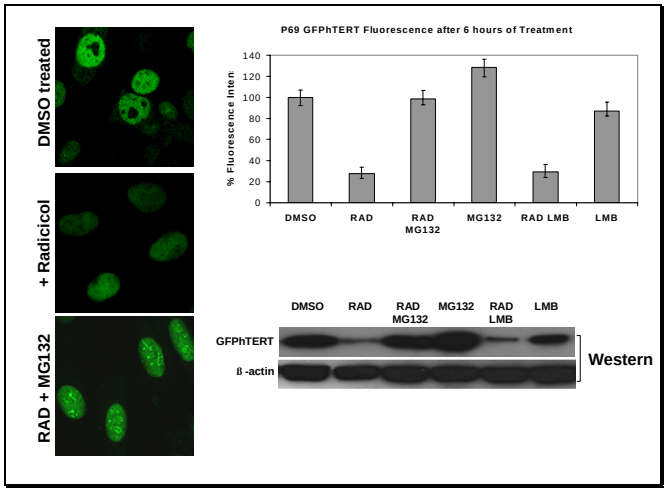

Figure 2: Nuclear fluorescence of GFPhTERT. Confocal microscopy shows GFPhTERT localized to the nucleoplasm (top left). Similar results occur with LMB, PP1, MG132 alone. After 1 hour, Radicicol (RAD) treatment begins to show the loss of signal from the nucleus (middle left). RAD and MG132 treatment together shows no loss of fluorescence and a punctuate aggregation around the nucleoli (bottom left). The top right panel is the quantitation of nuclear fluorescence intensity GFPhTERT after treatments with various compounds as measured by confocal microscopy. Bottom right panel shows the levels by Western of GFP-hTERT after the indicated treatments.

Like many Hsp90 client proteins, telomerase is degraded after Hsp90 inhibition. Specifically, Kim et al showed that telomerase was ubiquinated by MKRN1 then degraded by the proteasome (Kim et al., 2005). Thus, we initially postulated that a destabilized telomerase due to Hsp90 inhibition caused its nuclear exportation followed by cytoplasmic degradation. We envisioned a model in which the destabilized telomerase is phosphorylated by a Src kinase at Tyrosine 707 to signal its exportation through the 14-3-3 sigma/Exportin path, followed by ubiquination with an E3 ligase and degradation by the 26S proteasome in the cytoplasm. However, our preliminary data shows that inhibition of Exportin and hsp90 still caused the same loss of fluorescence intensity as compared to Hsp90 inhibition alone (Fig. 2). The combination treatment of RAD and proteasome inhibitor, MG132, remains fluorescent and nucleoplasmic, suggesting that degradation of telomerase occurs in the nucleus. In fact, telomerase appears to accumulate around the nucleoli into protein aggregates (Fig. 2 bottom left panel). These “aggresomes” have been described for other proteins and consist of chaperones, ubiquinated proteins and the 26S proteasome (Johnson et al., 1998). Western analysis shows that the protein levels of GFP-hTERT correspond to the quantitation of the loss of fluorescence after Hsp90 inhibition (compare top right panel for the quantification with the bottom right panel for the Western blot). Immunoprecipitation followed by Western analysis of the combination treatment (RAD and MG132) showed less degradation as compared to RAD alone, yet increased ubiquination as compared to control (Fig. 3). We also tested to determine if hTERT co-localized with 26S proteosome within the nucleus using immunofluorescence, and found that after treatment with RAD, GFP-hTERT and nuclear 26S were co-localized in RAD-treated cells after inhibition of degradation with the MG132 proteosome inhibitor (Fig. 4). Taken together, these data suggest that Hsp90 inhibition causes telomerase to be ubiquinated then degraded by proteasomes located within the nucleus. Review of the

literature shows the existence of a nuclear ubiquitin/proteasome pathway with all of the appropriate components as well as a few substrates and functions (von Mikecz, 2006).

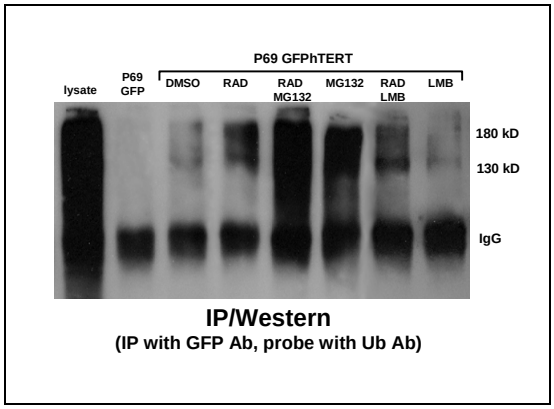

Figure 3. Ubiquination and degradation products of GFPhTERT after 2 hours of treatment. Cells were lysed and immunoprecipitated for GFP followed by Western for ubiquitin. Radicicol treatment shows high levels of degradation as compared to the other conditions. MG132 alone shows a slightly higher level of ubiquination as expected due to inhibition of normal GFPhTERT turnover. The band at 55 kD represents the IgG from IP. CTL is untreated control. Separate lanes on the same exposed film were spliced together to cut out unrelated experimental data.

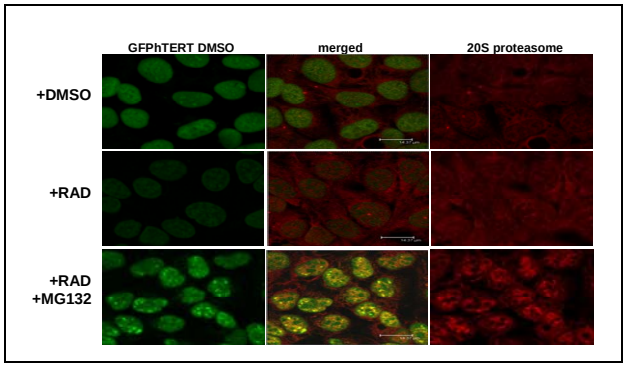

Figure 4. GFPhTERT Co-localizes with the Nuclear Proteasome after Inhibition (MG132) of RAD-Induced Degradation. Cells were treated with DMSO (control), RAD alone, or RAD+MG132 and tested for the co-localization of the GFP-hTERT with the 26S proteosome using immunofluorescence. Note minimal overlap in the RAD alone treated cells (merged panel) as most of the GFP-hTERT is degraded. The MG132 prevents the degradation, showing co-localization of GFP-hTERT with the 26S proteosome.

Cytoplasmic Degradation

Recent data from our lab suggested that a dominant-negative form of hTERT caused inhibition of telomerase activity, shortening of telomerase, and perhaps degradation in a variety of cell lines. In order to follow the degradation of hTERT, we created a GFP-DN-hTERT fusion protein and characterized cells expressing this construct (Fig. 5). We find that DN-hTERT induces telomere shortening and is partially degraded, as there is no significant difference in mRNA levels (Fig. 5, RT-PCR panel). Treatment of cells with the proteosome inhibitor, MG132, prevents degradation of DN-hTERT (Fig. 6, top and bottom panels), while the nuclear exportation inhibitor, LMB, prevents export of DN-hTERT to the cytoplasm (retained in the nucleus), subsequently preventing degradation (Fig. 6, top panel). Together, these data suggest that DN-hTERT is exported from the nucleus to the cytoplasm for degradation.

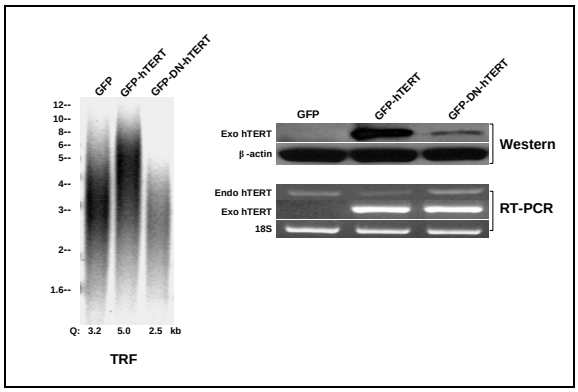

Figure 5. DN-hTERT Shortens Telomeres and is Partially Degraded. The terminal restriction fragment (TRF) length assay shows that telomeres are elongated in GFP-hTERT cells and shortened in GFP-DN-hTERT cells. Western analysis reveals that expression of the GFP-DN-hTERT is substantially lower than the wild-type GFP-hTERT, while RT-PCR indicates sufficient expression at the mRNA level, suggesting that DNhTERT is at least partially degraded.

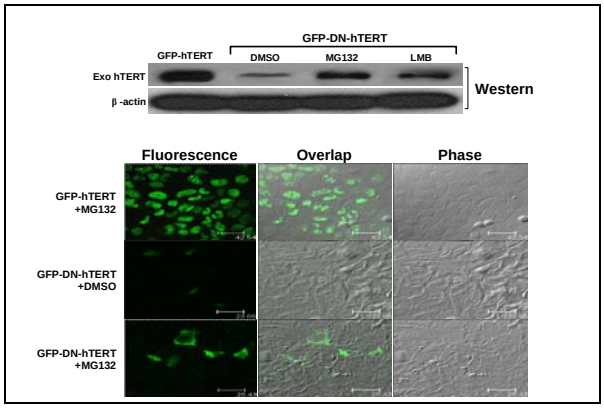

Figure 6. DN-hTERT is exported to the Cytoplasm and Degraded. Western analysis reveals that in the presence of MG132, DN-hTERT levels are increased compared to DMSO treatment, while the nuclear exportation inhibitor, LMB, which prevents DN-hTERT from exiting the nucleus, also allows accumulation of DN-hTERT. This is partially confirmed by immunofluorescence with the GFP-DN-hTERTwith the proteosome inhibitor, MG132, showing cytoplasmic expression and retention.

To more accurately assess whether DN-hTERT can physically cause the translocation of wild-type hTERT to the cytoplasm for degradation, we dually infected GFP-hTERT and the DN-hTERT without the GFP fusion. Thus, if DN-hTERT "drags" wild-type hTERT out of the nucleus, we should see GFP-hTERT in the cytoplasm. Figure 7 shows that normally, GFP-hTERT is cytoplasmic (in the absence of DN-hTERT), but when DN-hTERT is also expressed, the wild-type GFP-hTERT is predominantly cytoplasmic, indicating that one of the mechanisms for the dominant-negative effect of telomerase is translocation of wild-type to the cytoplasm, reducing its telomere maintenance function.

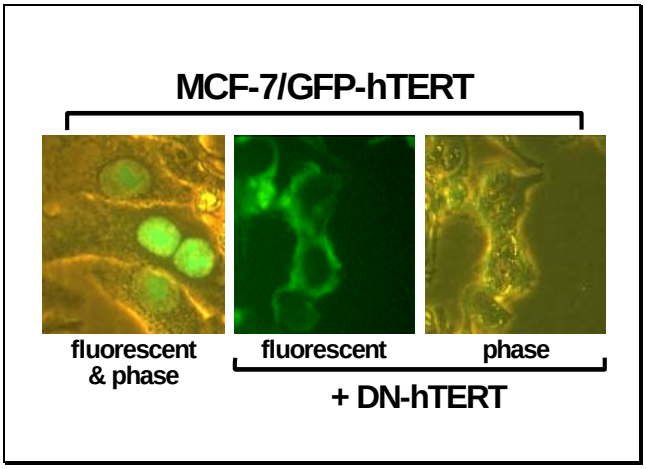

Figure 7. DN-hTERT causes translocation of GFP-hTERT from the nucleus to the cytoplasm. When GFPhTERT is stably expressed in MCF-7, it is localized to the nucleus (far left). In contrast, stable expression of the GFPDNhTERT in MCF-7 results in cytoplasmic localization (middle). Phase-contrast verifies the cytoplasmic fluorescence (right).

Figure 8 clearly shows that both the wild-type and dominant-negative hTERT are ubiquitinated (Fig. 8B) and degraded (Fig. 8A), presumably in the cytoplasm (see Fig. 7, with no effect on mRNA levels (Fig. 8A, RT-PCR). Collectively, our data indicate that DN-hTERT functions, at least partially, to inhibit the wild-type hTERT by translocation to the cytoplasm for degradation via a ubiquitin-mediated mechanism.

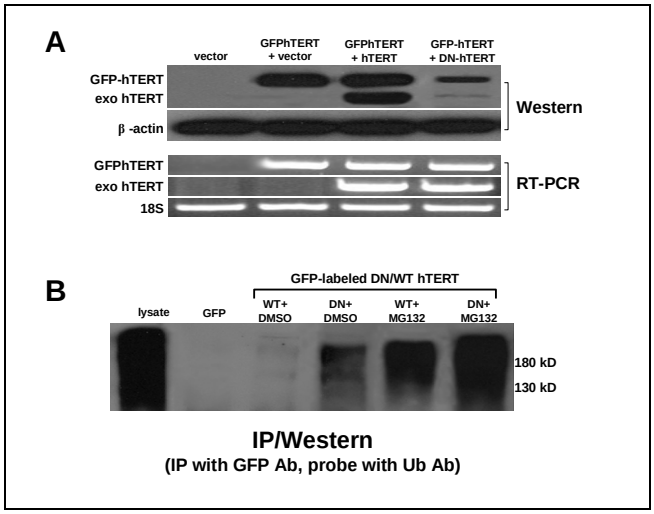

Figure 8. DN-hTERT Reduces Wild-Type hTERT Levels. (A) GFP-hTERT expressing cells were infected with vector, wild-type/untagged hTERT (+hTERT), or DN-hTERT and assessed for expression at both the RNA (RT-PCR) and protein levels. Expression of DN-hTERT together with GFP-hTERT induces the degradation of wild-type GFP-hTERT. **(B)** GFP-hTERT or GFP-DN-hTERT were immunoprecipitated with GFP antibodies and lysates subjected to Western analysis using a ubiquitin antibody, showing that the DN-hTERT is ubiquitinated like the wild-type hTERT.

Key Research Accomplishments

1-cell culture models of the GFP/telomerase fusion over-expression have been created, in mammary-related cell lines.

2-specific mechanisms related to telomerase stability and degradation have been identified and explored.

3-identification of a novel chaperone-mediated mechanism for degrading telomerase in the nucleus.

Recommended Changes to the Proposed Work Based on Additional Findings

None

Reportable Outcomes

Manuscripts

Elmore,L.W. and S.E.Holt. 2007. Telomerase inhibition as an adjuvant anti-cancer therapy: it's more than just a waiting game. Expert Opinion on Therapeutic Targets 11:427-430.

Di,X., R.Bellotti, A.T.Bright, E.Gaskins, J.Roberts, D.A.Gewirtz, S.E.Holt and L.W.Elmore. 2008. Induction of a senescence bystander effect in breast cancer cells: implications for treatment design and response. Cancer Biology and Therapy, in press.

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Abstracts/Presentations

Elmore,L.W., S.C.Henderson, A.T.Bright, X.Di., D.A.Gewirtz, and S.E.Holt. Telomere and Genome Stability, Villars-su-Ollon, Switzerland, August 2006.

Depcrynski,A., L.W.Elmore, and S.E.Holt. Women in Medicine and Science retreat, VCU, April 2007.

Liu,M.C., M.Idowu, K.Kimmelshue, S.E.Holt and L.W.Elmore,. Women in Medicine and Science retreat, VCU, April 2007.

Holt,S.E., M.Norris, S.Sircar, A.T.Bright, R.Winn, and L.W.Elmore. Telomeres and Telomerase, Cold Spring Harbor meeting, Cold Spring Harbor, NY, May 2007.

Depcrynski,A., L.W.Elmore, and S.E.Holt. Telomeres and Telomerase, Cold Spring Harbor meeting, May 2007.

Liu,M.C., M.Idowu, K.Kimmelshue, S.E.Holt and L.W.Elmore,. Telomeres and Telomerase, Cold Spring Harbor meeting, Cold Spring Harbor, NY, May 2007.

Nguyen,B.N., L.W.Elmore, and S.E.Holt. Telomeres and Telomerase, Cold Spring Harbor meeting, Cold Spring Harbor, NY, May 2007.

Francis,M.P., P.C.Sachs, G.L.Bowlin, L.W.Elmore, and S.E.Holt. The Stem Cell Summit, Boston, MA. October 2007.

Sachs,P.C., M.P.Francis, L.W.Elmore, and S.E.Holt. The Stem Cell Summit, Boston, MA. October 2007.

Invited Seminars

Holt,S.E. Keynote Speaker, Pugwash Conference, Richmond, VA. January 2008.

Holt,S.E. Department of Applied Medical Sciences, University of Southern Maine, Portland, ME. November 2007.

Holt,S.E. Department of Biochemistry and Molecular Biology, University of Virginia, September 2007.

Holt,S.E. Department of Biology, Maggie Walker Governor's School, Richmond, VA. March 2007.

Holt,S.E. Department of Biochemistry, Peking University, Beijing, China. March 2007.

Development of Cell Lines

We are have generated a variety of cell lines using the GFP-hTERT fusion protein (Wong et al., 2002) to be used to understand telomerase trafficking and regulation.

Funding Applied For

P.I.:	Shawn E. Holt, Ph.D.
Title:	Development of an In Vitro Breast Cancer Progression Model System using Primary Mammary Cells and In Vivo Selection
Agency:	Department of Defense Breast Cancer program (grant # **W81XWH-04-0551)**
Amount:	$486,000 total direct
Duration:	3/1/07-5/31/09 [DECLINED]

Conclusions

Overall, the use of specific telomerase antibodies has been limited, which in turn limits our ability to identify telomerase interacting proteins using immunoprecipitation. As a result of this setback, we have cloned and expressed a fusion protein of telomerase using the GFP tag, which provides us with antibodies and visualization of the telomerase enzyme. We have shown that telomerase is regulated via 2 distinct mechanisms related to hsp90-mediated degradation: in the nucleus and in the cytoplasm. As a result, we have expanded our proposal to defining the mechanisms of telomerase stability and cellular regulation, in addition to the discovery of telomerase associated proteins specific for breast cancer. Understanding the regulatory mechanisms related to telomeres and telomerase in mammary-related cells will facilitate the development of improved therapeutic strategies specifically targeting breast tumor cells.

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BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Shawn E. Holt, Ph.D.	Associate Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
The Colorado College, Colorado Springs, CO	B.A.	1985-1989	Biology
Texas A&M University, College Station, TX	Ph.D.	1989-1994	Genetics
The University of Texas Southwestern, Dallas, TX	Postdoc	1994-1998	Aging and Cancer

RESEARCH AND PROFESSIONAL EXPERIENCE:, INCLUDING GRANT SUPPORT. DO NOT EXCEED 3 PAGES.

A. Positions and Honors

Positions:

1998-2003	Assistant Professor, Department of Pathology and Department of Human Genetics, Virginia Commonwealth University/Medical College of Virginia, Richmond, VA
2003-present	Associate Professor, Department of Pathology and Department of Human Genetics, Virginia Commonwealth University, Richmond, VA
1998-present	Member, Massey Cancer Center, Virginia Commonwealth University, Richmond, VA
2002-present	Adjunct Faculty, Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, VA
2002-present	Member, Molecular Biology and Genetics Program, Virginia Commonwealth University, Richmond, VA
2003-present	Director, Graduate Studies and Education, Department of Pathology, Virginia Commonwealth University, Richmond, VA

Honors:

2000-2003	The V Foundation Scholars Program, Cary, NC
1996-1998	NRSA Fellowship, National Institute on Aging, while at UT Southwestern, Dallas, TX
1994	Outstanding Presenter, Research Symposium, Texas A&M University, College Station, TX
1994	Outstanding Student Government Member, Texas A&M University, College Station, TX
1988-1989	Dean's List, The Colorado College, Colorado Springs, CO
1988	Most Dedicated Football Player, The Colorado College, Colorado Springs, CO
1987	Rookie of the Year, Baseball, The Colorado College, Colorado Springs, CO
1985-1987	Outstanding College Students of America

B. Selected Peer-Reciewed Publications (over the past 4 years, from a total of 60)

Forsythe,H.L. J.L.Jarvis, J.W.Turner, L.W.Elmore, and **S.E.Holt.** 2001. Stable association of hsp90 and p23 with human telomerase. J Biol. Chem. **276**:15571-15574.

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Books and Book Chapters (from a total of 7)

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C. Research Support

Active

P.I.: Lawrence F. Povirk, Ph.D. (Co-I, 5% effort, Shawn E. Holt, Ph.D.)
Title: Tyrosyl-DNA phosphodiesterase and oxidative DNA damage
Agency: NIH
Amount: $1,125,000 total direct
Duration: 6/1/04-5/31/09

P.I.: Colleen K. Jackson-Cook, Ph.D. (Co-I, 10% effort, Shawn E. Holt, Ph.D.)

Title: Aging and Genomic Changes: Role of Environment/Genetics
Agency: NIH/NIEHS
Duration: 12/1/05-11/30/10

P.I.: Lynne W. Elmore, Ph.D.
Mentors: Shawn E. Holt, Ph.D. and David A. Gewirtz, Ph.D. (0% effort)
Title: Genomic instability and senescence in breast cancer
Agency: NIH (K-08, Temin Award)
Duration: 9/1/05-8/31/10

Completed

P.I.: Shawn E. Holt, Ph.D.
Title: Mechanisms of Prostate Cancer Transformation
Agency: Department of Defense
Duration: 12/18/01-12/17/05

P.I.: Kennon R. Poynter
Mentor: Shawn E. Holt, Ph.D.
Title: Mechanisms of telomerase inhibition using small inhibitory RNAs and induction of breast tumor cell sensitization
Agency: Department of Defense Breast Cancer program
Duration: 4/1/04-3/31/07

P.I.: Shawn E. Holt, Ph.D.
Title: Defining the regulation of telomerase through identification of mammary-specific telomerase interacting proteins
Agency: Department of Defense Breast Cancer program
Duration: 6/1/04-5/31/07